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RELATIONSHIPS OF THE HETERONCHOCLEIDIDS (*HETERONCHOCLEIDUS*, *EUTRIANCHORATUS* AND *TRIANCHORATUS)* AS INFERRED FROM RIBOSOMAL DNA NUCLEOTIDE SEQUENCE DATA

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***ABSTRACT.* –** The monogeneans, *Heteronchocleidus*, *Eutrianchoratus* and *Trianchoratus*, were postulated to be closely related based on their haptoral hard parts of three well-developed anchors and one vestigial anchor. In this present study, partial 28S rDNA sequences from 12 heteronchocleidids species (10 *Trianchoratus* spp. and two *Eutrianchoratus* spp.) were obtained. Phylogenetic trees generated based on 28S rDNA (from the present study and GenBank) using neighbour-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) analyses show that members of these three genera constitute a clade. Within this main monophyletic clade are the *Heteronchocleidus-Eutrianchoratus* and the *Trianchoratus* clades. This suggests that the ancestral form of these monogeneans (probably with three developed and one vestigial anchor and two connective bars) diverged into two lineages, the *Heteronchocleidus-Eutrianchoratus* clade with the retention of bars and the *Trianchoratus* clade with the complete loss of connective bars. The *Heteronchocleidus-Eutrianchoratus* lineage diverged into the *Eutrianchoratus* group with the loss of one bar and the *Heteronchocleidus* group with the retention of the two bars. These monogeneans were initially assigned to the Heteronchocleidinae Price, 1968 which is herein raised to family status, Heteronchocleididae. The anabantoids and channids, which are the fish hosts of heteronchocleidids, are also shown to be closely related based on the phylogenetic tree generated on their partial Cytochrome *b* sequences obtained from GenBank. The monophyly of the heteronchocleidids, their distribution patterns on the anabantoid and channid hosts and the relatedness of their fish hosts suggest that the ancestral heteronchocleidids could be present on the ancestral forms of the anabantoids and channids. The hosts could have acquired their heteronchocleidids through inheritance and/or host transfers. Whatever the method of acquisitions, the process of speciation and extinction of some of the heteronchocleidid monogeneans on the different anabantoid and channid hosts gave rise to the present-day distribution patterns of the heteronchocleidids. Although the monophyly of the heteronchocleidids has been established, the interrelationships of this clade to the other monogenean groups and their evolutionary history need further investigation.

***KEY WORDS.* –** *Heteronchocleidus, Eutrianchoratus, Trianchoratus*, Anabantoid, Channidae, Heteronchoclididae.

INTRODUCTION

Members of the genera *Heteronchocleidus* Bychowsky, 1957, *Eutrianchoratus* Paperna, 1969 and *Trianchoratus* Price & Berry, 1966 have three well-developed anchors and one vestigial anchor and similar types of sclerotized reproductive structures (copulatory organ and vagina). These monogeneans differ from one another in the number of connective bars (two bars in *Heteronchocleidus* spp., one bar in *Eutrianchoratus* spp. and no bar in *Trianchoratus* spp.) as well as in the shapes and sizes of the three well-developed anchors (Lim, 1986, 1989; Tan et al., 2010). To date, 10 species of *Heteronchocleidus*, nine species of *Eutrianchoratus* and 11 species of *Trianchoratus* have been described from 13 species of anabantoids and three species of channids from the Oriental biogeographical region of South China, Peninsular Malaysia and India and also from the Ethiopian region of Africa (Table 1). In Peninsular Malaysia, one species of *Heteronchocleidus*, four species of *Eutrianchoratus* and 10 species of *Trianchoratus* have

Table 1. List of *Heteronchocleidus*, *Eutrianchoratus* and *Trianchoratus* species from anabantoid and channid fishes.

Monogenean species	Host species[1]	Host family/subfamily	Locality	Reference
Heteronchocleidus buschkieli Bychowsky, 1957	*Macropodus opercularis*	Macropodinae	China	Bychowsky, 1957
H. buschkieli Bychowsky, 1957	*Macropodus chinensis*	Macropodinae	China	Tchang & Ji, 1980
H. buschkieli Bychowsky, 1957	*Trichopsis vittata*	Macropodinae	Malaysia	Lim, 1986
H. gracilis Mizelle & Kritsky, 1969	*Trichogaster labiosus* (*Colisa labiosa*)	Luciocephalinae	India	Mizelle & Kritsky, 1969
H. ctenopomae Paperna, 1969	*Ctenopoma kingsleyae*	Anabantidae	Ghana, Africa	Paperna, 1969
H. ouemensis Euzet & Dossou, 1975	*Ctenopoma kingsleyae, C. petherici*	Anabantidae	Africa	Euzet & Dossou, 1975
H. tuzetae Euzet & Dossou, 1975	*Ctenopoma kingsleyae, C. petherici*	Anabantidae	Africa	Euzet & Dossou, 1975
H. adjanohouni Euzet & Dossou, 1975	*Ctenopoma kingsleyae, C. petherici*	Anabantidae	Africa	Euzet & Dossou, 1975
H. magnihamatus Tchang & Ji, 1980	*Macropodus chinensis*	Macropodinae	China	Tchang & Ji, 1980
H. asymmetricus Majumdar et al., 1988	*Trichogaster lalius* (*Colisa lalia*)	Luciocephalinae	India	Majumdar et al., 1988
H. stunkardi Majumdar et al., 1988	*Trichogaster lalius* (*Colisa lalia*)	Luciocephalinae	India	Majumdar et al., 1988
H. lucknowensis Agarwal & Bhatnagar, 1997	*Trichogaster fasciata* (*Colisa fasciata*)	Luciocephalinae	India	Agarwal & Bhatnagar, 1997
Eutrianchoratus magnum Paperna, 1969	*Parachanna obscura* (*Ophicephalus obscurus*)	Channidae	Ghana, Africa	Paperna, 1969
E. minutus Paperna, 1969	*Parachanna obscura* (*Ophicephalus obscurus*)	Channidae	Ghana, Africa	Paperna, 1969
E. imbachi Dossou & Euzet, 1984	*Parachanna obscura* (*Ophicephalus obscurus*)	Channidae	Benin, Africa	Dossou & Euzet, 1984
E. cleithrium Lim, 1989	*Belontia hasseltii*	Belontiinae	Malaysia	Lim, 1989
E. distensigenitalis Lim, 1989	*Belontia hasseltii*	Belontiinae	Malaysia	Lim, 1989
E. fusiformis Lim, 1989	*Belontia hasseltii*	Belontiinae	Malaysia	Lim, 1989
E. inequalis Lim, 1989	*Belontia hasseltii*	Belontiinae	Malaysia	Lim, 1989
E. chibami Bilong Bilong et al., 1994	*Parachanna obscura*	Channidae	Cameroon, Africa	Bilong Bilong et al., 1994
E. malleus Bilong Bilong et al., 1994	*Parachanna obscura*	Channidae	Cameroon, Africa	Bilong Bilong et al., 1994
Trianchoratus acleithrium Price & Berry, 1966	*Helostoma temminckii* (*H. rudolfi*)	Helostomatidae	Southeast Asia[2]	Price & Berry, 1966
T. acleithrium Price & Berry, 1966	*Helostoma temminckii*	Helostomatidae	Malaysia	Lim, 1986
T. grandis Lim, 1986	*Anabas testudineus*	Anabantidae	Malaysia	Lim, 1986
T. gussevi Lim, 1986	*Anabas testudineus*	Anabantidae	Malaysia	Lim, 1986
T. gussevi Lim, 1986	*Anabas testudineus*	Anabantidae	China	Ding & Liao, 2005
T. parvulus Lim, 1986	*Anabas testudineus*	Anabantidae	Malaysia	Lim, 1986
T. kearni Agrawal & Bhatnagar, 1994	*Anabas testudineus*	Anabantidae	India	Agrawal & Bhatnagar, 1994
T. leerium Lim, 1986	*Trichopodus leerii* (*Trichogaster leerii*)	Luciocephalinae	Malaysia	Lim, 1986
T. trichogasterium Lim, 1986	*Trichopodus trichopterus* (*Trichogaster trichopterus*)	Luciocephalinae	Malaysia	Lim, 1986
T. pahangensis Lim, 1986	*Channa striata*	Channidae	Malaysia	Lim, 1986
T. malayensis Lim, 1986	*Channa lucius*	Channidae	Malaysia	Lim, 1986
T. ophicephali Lim, 1986	*Channa lucius*	Channidae	Malaysia	Lim, 1986
T. longianchoratus Tan & Lim, 2009	*Channa lucius*	Channidae	Malaysia	Tan & Lim, 2009

[1] Valid names following FishBase (Froese & Pauly 2010) and Töpfer & Schindler (2009); in parentheses, synonym names used in the original sources.

[2] Fish specimens were obtained from Cordell Farm Supply Co., Milledgeville, Georgia, USA and listed as from Sumatra, Borneo, Java, Malaya and Thailand.

been recorded from six species of anabantoids and two species of channids (Lim, 1986, 1989; Tan & Lim, 2009) whilst four species of *Heteronchocleidus* and five species of *Eutrianchoratus* are found on two species of African anabantoids and one species of channid, respectively (Table 1). In India, there are four species of *Heteronchocleidus* and one species of *Trianchoratus* from four species of anabantoids whereas in China, thus far only two species of *Heteronchocleidus* and one species of *Trianchoratus* were recorded on three species of anabantoids (Table 1). Based on morphological characteristics, these monogeneans were considered to be related and included in the subfamily Heteronchocleidinae Price, 1968 in Dactylogyridae Bychowsky, 1937 by Price (1968) and Paperna (1969), in the subfamily Ancyrocephalinae Bychowsky, 1957 and family Ancyrocephalidae Bychowsky, 1937 by Gusev (1978) and later in the subfamily Heteronchocleidinae and family Ancyrocephalidae by Lim (1986, 1989, 1998) (see later).

It will be interesting to see whether the evolutionary relationships constructed based on morphologies by Lim (1987) will be supported by their molecular data. To date molecular data (28S rDNA) are only known from one *Trianchoratus* species and one *Heteronchocleidus* species (Ding & Liao, 2005) (Table 2). To determine the phylogenetic relationships of the *Heteronchocleidus*, *Eutrianchoratus* and *Trianchoratus*, partial sequences of the D1 domain of 28S rDNA are here obtained from 10 *Trianchoratus* spp. and two *Eutrianchoratus* spp. from Malaysian anabantoids and channids (Table 2).

Evolutionary relationships of the hosts may assist in understanding the evolutionary history of the parasites (Szidat, 1956 ; Fahrenholz, 1913; Jovelin & Justine, 2001). According to Fahrenholz's rule, the distribution of the morphologically related monogeneans (*Heteronchocleidus, Eutrianchoratus* and *Trianchoratus*) on the anabantoids and channids indicate that these two fish groups could be phylogenetically related (Lim, 1986, 1989). The anabantoids and channids have been considered to be related based on their morphologies (Roberts, 1989; Ng & Lim, 1990; Nelson, 1994). The molecular phylogenetic relationships of the anabantoids and channids have been analysed separately (Li et al., 2006; Rüber et al., 2006; Adamson et al., 2010). To date, there has been no effort to determine the relationships between the anabantoids and channids. DNA sequences of the anabantoids and channids are available in GenBank and these molecular data together with those of the catfish families and cyprinids from GenBank (Table 3) are here used to construct a relationship tree to determine if molecular data support or refute their relationships based on morphological characteristics.

MATERIAL AND METHODS

***Collection of hosts and parasites.* –** In this study, six *Trianchoratus* species were collected from four species of anabantoids [*Helostoma temminkii* (Cuvier), *Anabas testudineus* (Bloch), *Trichopodus leerii* (Bleeker) and *T. trichopterus* (Pallas)], four *Trianchoratus* species were from two species of channids [*Channa lucius* (Cuvier) and *C. striata* (Bloch)] and two species of *Eutrianchoratus* were from *Belontia hasselti* (Cuvier) (see Table 2). The fish hosts were collected from Peninsular Malaysia. The monogeneans were gently dislodged from freshly removed gills of the hosts and preserved in 75% ethanol. Parasites were removed from ethanol, transferred individually using fine pipette onto glass slide with a drop of distilled water and covered with a cover slip and examined and identified under a light microscope equipped with phase contrast and Leica image analysis software (Qwin Plus).

***DNA extraction, PCR and DNA sequencing.* –** The identified parasite was removed from the glass slide and put individually in separate 0.5 ml Eppendorf tube and genomic DNA was extracted using DNEasy extraction kit from QIAGEN. The extracted DNA (5 µl) was used as template in a PCR reaction to amplify the partial D1 domain of the 28S rDNA, using primers C1 (5'-ACCCGCTGAATTTAAGCAT-3') and C2 (5'-CTCTCTYTYCAAAGTTCTTTTC-3') (Justine et al., 2002). The PCR reaction (50 µl) was performed in 1.5 mM $MgCl_2$, PCR buffer (Fermentas), 200 µM of each deoxyribonucleotide triphosphate, 1.0 µM of each PCR primer, and 1 U of Taq polymerase (Fermentas) in a thermocycler (Biometra) using the following conditions: an initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 10 min. An aliquot (10 µl) from the amplicon was electrophoresed in a 1.3% agarose gel, stained with ethidium bromide and viewed under a UV illuminator. The remaining 40 µl of each amplicon was purified using DNA purification kit (QIAGEN) and subjected to automated DNA sequencing (ABI 3730 DNA Sequencer, First Base Laboratories) using the same primers as used for PCR amplification. A total of 12 species of heteronchocleidids were sequenced (Table 2).

***Analyses of DNA data.* –** DNA sequences from 14 species of heteronchocleidids (12 from present study, two from GenBank) and 18 species of other freshwater dactylogyrideans (from GenBank) (*Ancyrocephalus paradoxus*, *Bychowskyella pseudobagri*, *Quadriacanthus kobiensis*, *Cornudiscoides proximus*, *Thaparocleidus* spp., *Pseudodactylogyrus* spp. *Dactylogyroides longicirrus* and *Dactylogyrus* spp.) were analysed with *Diplectanum* spp. (Diplectanidae Bychowsky, 1957) and *Gyrodactylus* spp. (Gyrodactylidea Bychowsky, 1937) (from GenBank) as outgroups (see Table 2). The 38 DNA sequences obtained from the present study (12 sequences) and GenBank (26 sequences) were edited and aligned with Clustal X (Thompson et al., 1997) using default parameter and verified/edited visually by BioEdit version 7.0.5.3 (Hall, 1999). Relationship trees were constructed using maximum parsimony (MP), neighbor-joining (NJ), maximum likelihood (ML) and Bayesian inference (BI). MP analysis was performed using PAUP*4.0b10 (Swofford, 2002) where full heuristic searches were conducted with 10 random sequence additions and tree bisection-reconnection (TBR) branch swapping. Prior to NJ, ML and BI analyses, sequence alignment was tested for best-fit model of

Table 2. List of monogenean species used in this study with their host species, locality and GenBank accession numbers. (*present study) (**names used in GenBank).

Monogenean species	Host species	Locality	GenBank
Dactylogyridea Bychowsky, 1937			
Heteronchocleididae Price, 1968			
Trianchoratus malayensis	*Channa lucius*	Peninsular Malaysia	HQ719218*
Trianchoratus pahangensis	*Channa lucius*	Peninsular Malaysia	HQ719219*
Trianchoratus longianchoratus	*Channa lucius*	Peninsular Malaysia	HQ719220*
Trianchoratus ophicephali	*Channa striata*	Peninsular Malaysia	HQ719215*
Trianchoratus acleithrium	*Helostoma temminckii*	Peninsular Malaysia	HQ719214*
Trianchoratus leerium	*Trichopodus leerii* (*Trichogaster leerii***)	Peninsular Malaysia	HQ719216*
Trianchoratus trichogasterium	*Trichopodus trichopterus* (*Trichogaster trichopterus***)	Peninsular Malaysia	HQ719217*
Trianchoratus gussevi	*Anabas testudieus*	Peninsular Malaysia	HQ719221*
Trianchoratus gussevi CHN	*Anabas testudieus*	Hainan, China	AY841875
Trianchoratus parvulus	*Anabas testudieus*	Peninsular Malaysia	HQ719223*
Trianchoratus grandis	*Anabas testudieus*	Peninsular Malaysia	HQ719222*
Eutrianchoratus inequalis	*Belontia hasseltii*	Peninsular Malaysia	HQ719225*
Eutrianchoratus cleithrium	*Belontia hasseltii*	Peninsular Malaysia	HQ719224*
Heteronchocleidus buschkieli	*Macropodus opercularis*	Guangdong, China	AY841876
Dactylogyridae Bychowsky, 1933			
Dactylogyrus pekinensis	*Megalobrama amblycephala*	Guangdong, China	EF100535
Dactylogyrus quanfami	*Cirrhinus moliorella*	Guangdong, China	EF100536
Dactylogyrus lamellatus	*Ctenopharyngodon idellus*	Yunnan, China	EF100533
Dactylogyrus hypophalmichthys	*Hypophthalmichthys molitrix*	Chongqing, China	EF100532
Dactylogyrus parabramis	*Megalobrama terminalis*	Guangdong, China	EF100534
Dactylogyroides longicirrus	*Systomus sophore* (*Puntius sophore***)	India	GU903482
Pseudodactylogyridae Le Brun, Lambert & Justine, 1986			
Pseudodactylogyrus bini	*Anguilla anguilla*	Austria	AJ969949
Pseudodactylogyrus anguillae	*Anguilla anguilla*	Slovak Republic	AJ969950
Pseudodactylogyrus sp. UK	*Anguilla anguilla*	United Kingdom	AF382057
Pseudodactylogyrus sp. XHY	*Anguilla anguilla*	China	EF100540
Ancyrocephalidae Bychowsky, 1937			
Ancyrocephalus paradoxus	*Stizostedion lucioperca*	Czech Republic	AJ969952
Ancylodiscoididae Gusev, 1961			
Quadriacanthus kobiensis	*Clarias fuscus*	Guangdong, China	EF100545
Bychowskyella pseudobagri	*Pseudobagrus fulvidraco*	Guangdong, China	EF100541
Cornudiscoides proximus	*Mystus vittatus*	India	GQ925913
Thaparocleidus magnicirrus	*Silurus asotus*	Guangdong, China	EF100549
Thaparocleidus obscura	*Silurus asotus*	Chongqing, China	EF100551
Thaparocleidus mutabilis	*Silurus asotus*	Guangdong, China	EF100550
Thaparocleidus omegavagina	*Silurus asotus*	Guangdong, China	EF100552
Outgroups			
Diplectanidae Bychowsky, 1957			
Diplectanum penangi	*Lates calcarifer*	Hainan, China	DQ054821
Diplectanum grouperi	*Epinephelus coioides*	Guangdong, China	AY553628
Diplectanum umbrinum	*Johnius amblycephalus*	Guangdong, China	EF100560
Gyrodactylidae van Beneden & Hesse, 1863			
Gyrodactylus salaris	*Salmo salar*	Norway	FJ971996
Gyrodactylus derjavini	*Oncorhynchus mykiss*	Denmark	FJ971994
Gyrodactylus macracanthus	*Misgurnus anguillicaudatus*	Australia	FJ971995

nucleotide substitution using Akaike Information Criterion as implemented by Modeltest 3.7 (Posada & Crandall, 1998). NJ and ML relationships trees were constructed using PAUP*4.0b10 under the selected best-fit model and parameters with TBR branch swapping. Bayesian analysis was performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) based on the selected best-fit model and parameters, using four Markov Chain Monte Carlo (MCMC) chains, one cold and three heated. Bootstrap procedure (for NJ, MP, and ML) and posterior probabilities (for BI) were performed to assess the robustness of the inferred relationships. For NJ and MP, bootstrapping were conducted with 1000 replications while for ML, 100 replications were performed with heuristic searches due to long computational time. Relationship trees were displayed and edited using TreeView 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html/).

Table 3. List of partial Cytochrome *b* sequences from anabantoids, channids, silurids, bagrids, clariids and cyprinids (outgroup) used to infer relationships of fish species. *Valid names as in FishBase (Froese & Pauly 2010), Töpfer & Schindler (2009) and Ng & Tan (1999); in parentheses, names used in GenBank.

Host family	Host species*	GenBank accession number
Osphronemidae	*Belontia hasseltii*	AY763743
	Belontia signata	AY763744
	Betta macrostoma	AF519694
	Betta splendens	AF519689
	Trichogaster fasciata (*Colisa fasciata*)	AY763745
	Trichogaster labiosus (*Colisa labiosa*)	AY763746
	Trichogaster lalius (*Colisa lalia*)	AY763747
	Ctenops nobilis	AY763748
	Luciocephalus pulcher	AY763749
	Luciocephalus sp.	AY763750
	Macropodus spechti (*Macropodus concolor*)	AY763760
	Macropodus opercularis	AF519698
	Malpulutta kretseri	AF519700
	Osphronemus goramy	AY763768
	Osphronemus septemfasciatus	AY763769
	Parasphaerichthys lineatus	AY763751
	Parasphaerichthys ocellatus	AY763752
	Parosphromenus ornaticauda	AY763762
	Parosphromenus paludicola	AY763763
	Pseudosphromenus cupanus	AF519699
	Pseudosphromenus dayi	AY763764
	Sphaerichthys osphromenoides	AY763754
	Sphaerichthys selatanensis	AY763755
	Trichopsis pumila	AY763765
	Trichopsis schalleri	AY763766
	Trichopsis vittata	AF519697
	Trichopodus pectoralis (*Trichogaster pectoralis*)	AY763758
	Trichopodus leerii (*Trichogaster leerii*)	AF519695
	Trichopodus trichopterus (*Trichogaster trichopterus*)	AY763759
	Trichopodus microlepis (*Trichogaster microlepis*)	AY763757
Anabantidae	*Anabas testudineus*	AY763727
	Ctenopoma acutirostre	AY763728
	Ctenopoma kingsleyae	AY763729
	Ctenopoma petherici	AY763733
	Microctenopoma ansorgii	AY763736
	Microctenopoma fasciolatum	AY763738
	Sandelia capensis	AY763741
Helostomatidae	*Helostoma temminckii*	AY763742
Channidae	*Channa asiatica*	AF480933
	Channa bleheri	AY763770
	Channa lucius	GU288553
	Channa micropeltes	GU288556
	Channa maculata	FJ415743
	Channa marulia	AY763771
	Channa striata	GU288567
	Parachanna obscura	AY763772
Siluridae	*Kryptopterus minor*	AY458895
	Silurus asotus	DQ119376
	Wallago leerii	DQ119387
	Silurichthys schneideri	DQ119430
	Ompok bimaculatus	DQ119433
	Hemisilurus mekongensis	DQ119392
	Ompok miostoma	DQ119435
Bagridae	*Horabagrus nigricollaris*	HM579857
	Mystus sp.	AY458893
	Mystus bocourti	EU490912
	Bagrichthys macropterus	DQ119455
	Pelteobagrus argentivittatus (*Leiocassis argentivittatus*)	AY912443
	Pseudobagrus tenuis	AY912384

Table 3. Cont'd.

Host family	Host species*	GenBank accession number
Clariidae	*Clarias gariepinus*	DQ646372
	Heterobranchus longifilis	DQ646349
	Clarias batrachus	DQ119486
	Clarias nieuhofii (*Prophagorus nieuhofii*)	DQ119377
	Clarias liocephalus	DQ646353
	Gymnallabes typus	DQ119368
Cyprinidae (outgroup)	*Ctenopharyngodon idella*	AF420424
	Cyclocheilichthys janthochir	EU241418
	Systomus lineatus (*Puntius lineatus*)	EU241457
	Barbus rebeli	AF090791

To determine the relationships of the two groups of fish hosts (anbantoids and channids) the partial Cytochrome *b* sequences of 38 anabantoids, eight channids, seven silurids, six bagrids, six clariids and four cyprinids (as outgroups) were obtained from GenBank (Table 3) and Bayesian inference analysis as described above was used to construct the relationship tree (Fig. 3).

RESULTS

***Monogeneans.* –** In the current sequence alignment of the partial 28S rDNA from 38 monogenean species (12 from the present study and 26 from GenBank) (Table 2), there are 308 alignable positions containing 162 variable sites and of these, 138 sites are considered parsimony informative. Based on Modeltest 3.7, the current dataset of aligned sequences resulted in the best likelihood score for the general time reversible model with invariable sites and rate heterogeneity (GTR+I+G). Base frequencies are unequal where A=0.2435, C=0.2066, G=0.2674, T=0.2826 and the estimated proportion of invariable sites (pinvar) is 0.2663. The rate matrix (rmat) for the selected substitution model is [A-C]=0.7897, [A-G]=2.5473, [A-T]=2.0433, [C-G]=0.2462, [C-T]=3.8551, [G-T]=1.0000. The shape parameter of the gamma distribution is α = 0.6439. This model and parameters are used in NJ, ML and BI analyses.

All the relationships trees from NJ, MP, ML and BI (Figs. 1 & 2) show that species from the genera *Heteronchocleidus*, *Eutrianchoratus* and *Trianchoratus* form a monophyletic clade, which is distinct from the other clades formed by Pseudodactylogyridae (*Pseudodactylogyrus* spp.), Dactylogyridae (as represented by *Dactylogyrus* and *Dactylogyroides*) and Ancylodiscoididae (*Thaparocleidus*, *Cornudiscoides*, *Bychowskyella*, *Quadriacanthus*). The heteronchocleidid clade is found to be more related to the pseudodactylogyrid and dactylogyrid clades than to the ancylodiscoidid clade (see Lim et al., 2001) (Figs. 1 & 2).

Within the heteronchocleidid clade, there are two clades: *Heteronchocleidus-Eutrianchoratus* clade (Clade 1 – possessing one to two connective bars) and the *Trianchoratus* clade (Clade 2 – without connective bar) (Figs. 1 & 2). Within the *Trianchoratus* clade, it is observed that the *Trianchoratus* spp. are grouped according to their host groups: *Trianchoratus* spp. from the channids form a group (Group 1) distinct from the *Trianchoratus* spp. of the anabantoids (Group 2) (see Figs. 1 & 2). This indicates that *Trianchoratus* species from related host species are more related, suggesting that speciation has occurred within their respective host groups.

***Fish host.* –** The relationship tree for the fish hosts generated from Bayesian analysis using partial Cytochrome *b* sequences shows that the anabantoids (Osphronemidae, Helostomatidae, Anabantidae), channids and catfishes (Siluridae, Bagridae, Clariidae) form distinct separate clades (Fig. 3). The anabantoids and channids form sister groups which indicate that they are more closely related to each other than to other fish families (Fig. 3). This supports the relatedness of the anabantoids and channids postulated by various ichthyologists based on the similar morphological characteristics such as possessing accessory breathing organs and ecological habitats (Nelson, 1994; Lim, 1997). In fact there is a probable overlap in the divergence time of the African and Asian anabantoids and African and Asian channids which was estimated to be c. 30–87 Ma and c. 40–50 Ma, respectively (Rüber et al., 2006, Adamson et al., 2010).

DISCUSSION

***Relationships of members of the Heteronchocleididae Price, 1968.* –** The phylogenetic trees constructed based on 28S rDNA (Figs. 1 & 2) indicate that the *Heteronchocleidus*, *Eutrianchoratus* and *Trianchoratus* species form a monophyletic clade and in agreement with the relationships based on morphological characteristics proposed by Lim (1986, 1987, 1989). Lim (1987) noted the possibility of raising Heteronchocleidinae to familial status and the phylogenetic trees from the present study support this move. We propose herein to raise the status of Heteronchocleidinae to Heteronchocleididae Price, 1968.

The phylogenetic trees (Figs. 1 & 2) suggest that the ancestral monogenean of the heteronchocleidids diverged into the *Heteronchocleidus-Eutrianchoratus* clade (Clade 1) (with one to two connective bars) and the *Trianchoratus* clade (Clade 2) (without connective bars). The *Heteronchocleidus-Eutrianchoratus* clade subsequently split into two groups: the *Heteronchocleidus* group with the retention of the two bars and *Eutrianchoratus* spp. with the loss of one bar (Figs. 1 & 2). This evolutionary pathway of the heteronchocleidids based on current molecular data is different from that proposed

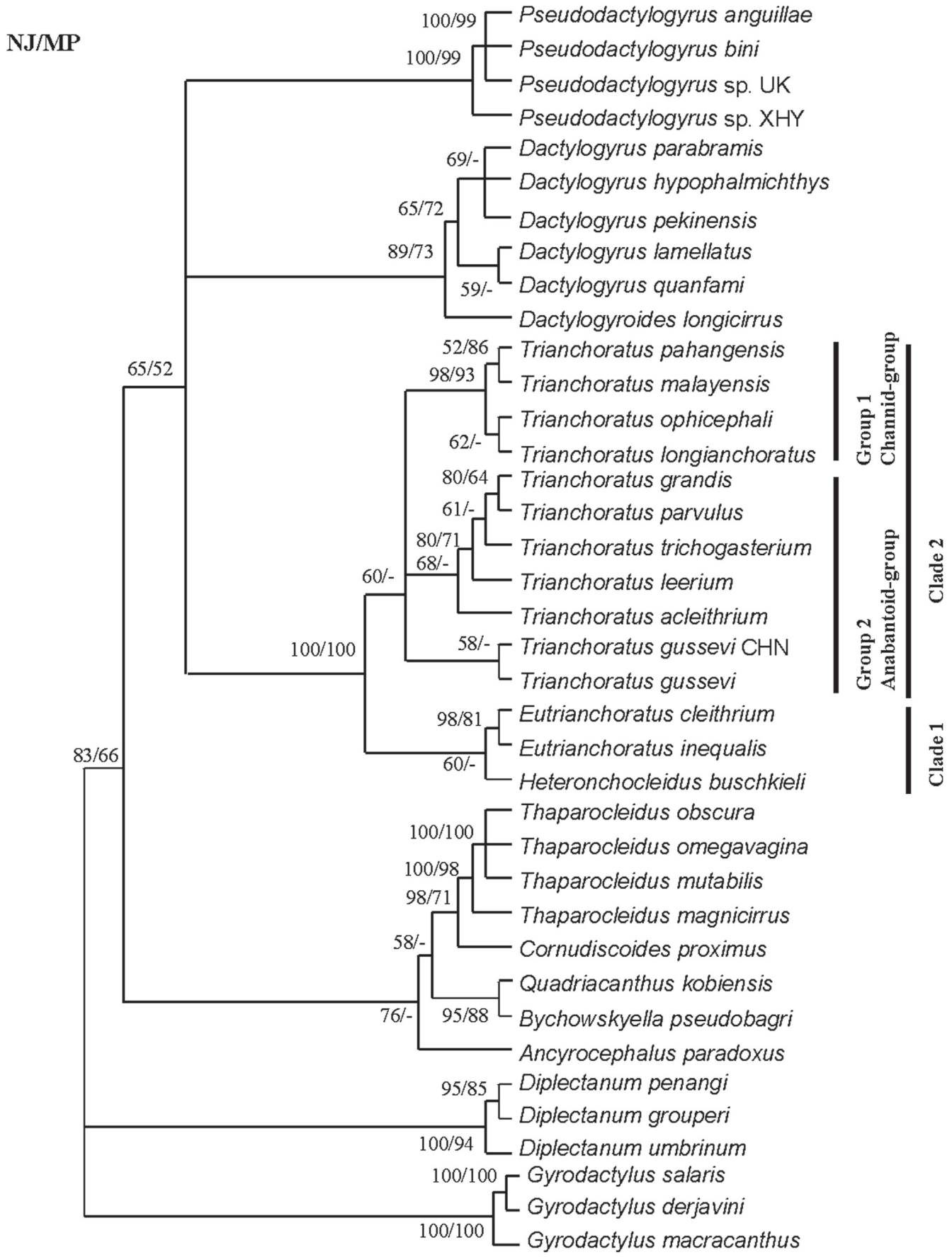

Fig. 1. Neighbour joining (NJ) tree constructed by PAUP* using partial 28S rDNA sequences (D1 domain) with *Diplectanum* spp. and *Gyrodactylus* spp. as outgroups. Percentages of the bootstrap values for neighbour joining (NJ)/maximum parsimony (MP) (NJ & MP=1,000 replicates) are shown along the branches. Bootstrap values lower than 50 are given as dashes (-).

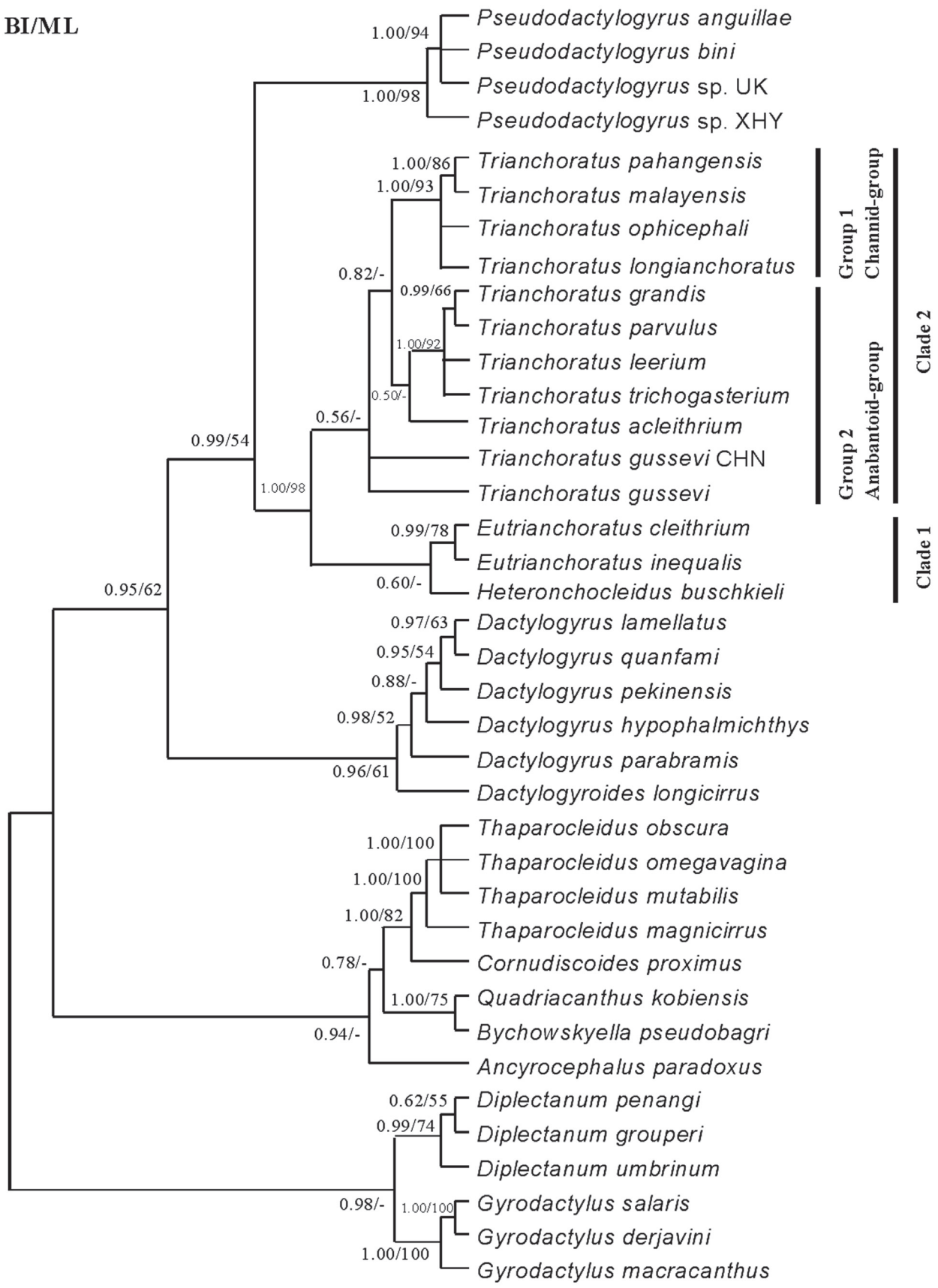

Fig. 2. Bayesian consensus tree generated from partial 28S rDNA sequences (D1 domain) with *Diplectanum* spp. and *Gyrodactylus* spp. as outgroups. Values shown at each node refer to Bayesian (BI) posterior probabilities/maximum likelihood (ML) percentages of the bootstrap values with 100 replicates. Bootstrap values lower than 50 are given as dashes (-).

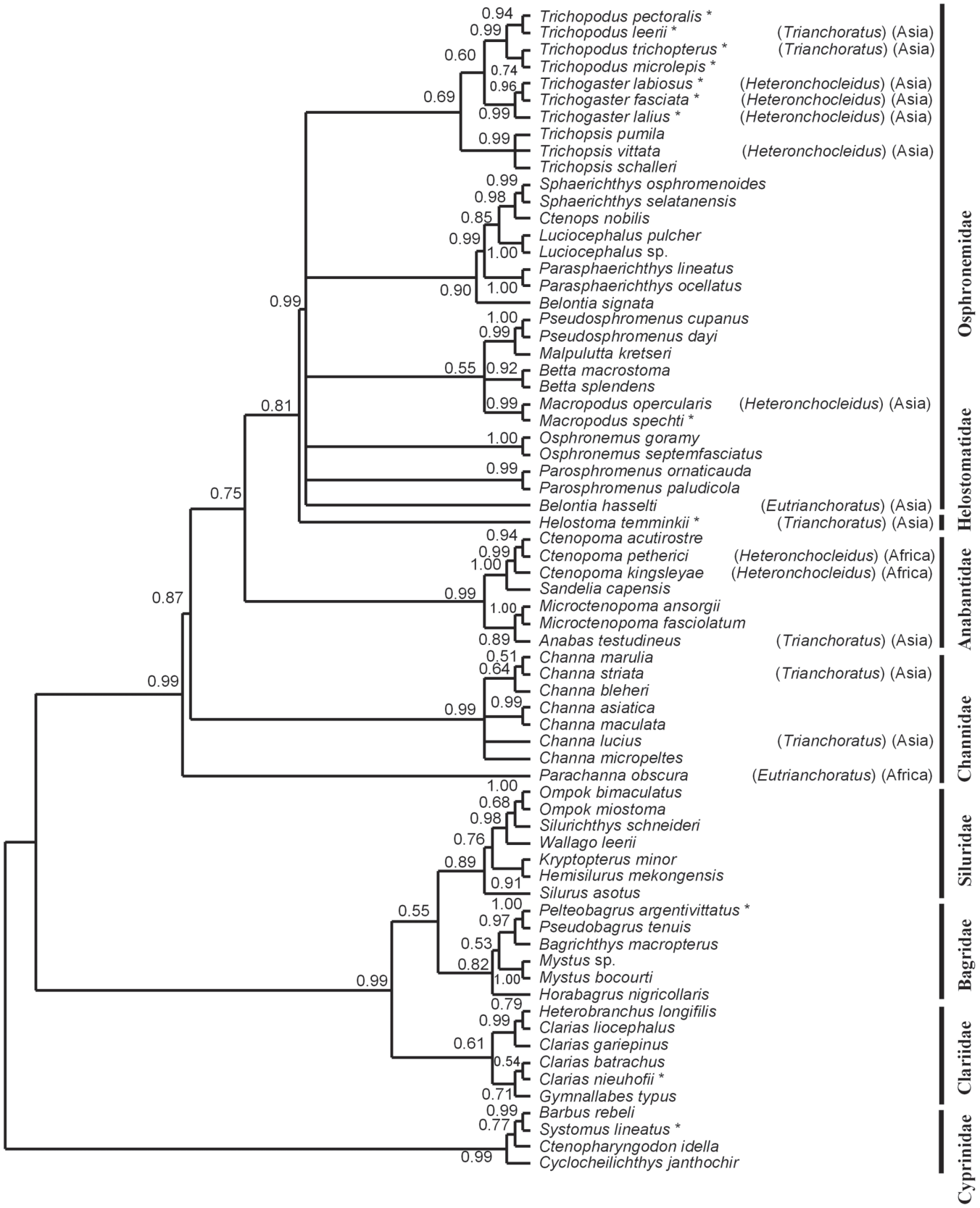

Fig. 3. Bayesian consensus tree for the anabantoids, channids and catfishes (silurids, bagrids, clariids) obtained using partial Cytochrome *b* sequences with cyprinids as outgroup. The heteronchocleidids genera present on the anabantoids and channids are shown with their geographical areas. Values shown at each node refer to Bayesian posterior probabilities. (*refer to Table 3 for names used in GenBank).

by Lim (1987) based on morphological characteristics. Lim (1987) suggested that the ancestral forms of the heteronchocleidids (probably with three developed anchors, one vestigial anchor and two connective bars) gave rise to the *Heteronchocleidus* group with the retention of two bars and to the *Eutrianchoratus -Trianchoratus* group with the loss of one bar which later diverged giving rise to the *Eutrianchoratus* group with the retention of the single bar and the *Trianchoratus* group with complete loss of the bar. In this study DNA sequences of only two *Eutrianchoratus*, one *Heteronchocleidus* and 10 *Trianchoratus* spp. (there are two sequences for *T. gussevi*, see Table 2) were available and more data is required to determine which of the two evolutionary pathways were employed by the ancestral heteronchocleidids.

***Interrelationships of Heteronchocleididae.* –** Lim (1987) suggested that heteronchocleidids are morphologically more closely related to other four anchor monogeneans, while the present analysis (Figs. 1 & 2) seems to link the heteronchocleidids (three developed and one vestigial anchors) to the pseudodactylogyrids (two anchors) and dactylogyrids (two anchors and two needles). The current phylogenetic trees seem to indicate the plasticity of the haptoral hard parts in these three monogenean taxa. The relationships shown could be due to the low numbers of monogenean groups being used in this particular analysis which masks actual relationships (see Pollock et al., 2002). Therefore although the present analysis confirms the monophyletic relationships of the heteronchocleidids (*Heteronchocleidus, Eutrianchoratus* and *Trianchoratus* spp.), molecular data from more monogenean taxa is needed before the interrelationships among the heteronchocleidids and the other monogenean groups can be properly assessed. The need for more taxa data versus more characters have been well debated by Hillis et al. (2003).

***Resolving the anabantoid and channid relationships and heteronchocleidids distribution.* –** The close relationships of the anabantoids and channids as indicated by the phylogenetic tree (Fig. 3) supports Fahrenholz's rule (Fahrenholz, 1913) that related parasite species are found on related host species. To date, there has been no agreement as to the centre of origin and dispersion of the anabantoids and channids. Li et al. (2006) and Lim (1997) suggested the possibilities that the channids originated and evolved in Gondwanaland, based on fish molecular data and monogenean distribution data, respectively. Adamson et al. (2010), on the other hand, suggested that the ancestor of the channids evolved in central Asia to form the *Parachanna* (Teugels & Daget) and *Channa* (Scopoli) groups which subsequently migrated to Africa and Asia, respectively. Rüber et al. (2006) suggested that the centre of origin and dispersion of the anabantoids could either be in Africa or in Asia.

Analysis of the host-heteronchocleidid distribution patterns revealed that each host species harbours only one heteronchocleidid genus and there seems to be no specificity of any of the three heteronchocleidids to any one host species or groups (Table 1). The distribution patterns of the *Heteronchocleidus* (on Chinese *Macropodus* (Linnaeus), Malaysian *Trichopsis* (Cuvier) and African *Ctenopoma* (Peters)), *Eutrianchoratus* (on Malaysian *Belontia* and African *Parachanna*) and *Trianchoratus* (on Malaysian and Indian anabantoids, Malaysian and Thai *Helostoma* and Malaysian channids) suggest that the ancestral form of the heteronchocleidids could be present on both the ancestral anabantoids and channids through inheritance or even through host transfer very early in the evolutionary history of the two host lineages. If we accept that host-transfer had taken place in the evolution of the heteronchocleidids, then the pertinent question is: which is the original host of the heteronchocleidids? It is difficult to say with any certainty whether the original hosts of the heteronchocleidids are the anabantoids, which possess all the presently known heteronchocleidid groups, or the channids, with only two heteronchocleidid groups. The general disagreement as to the centre of origin and dispersion of the anabantoids and channids and our current inability to ascertain the original hosts of the heteronchocleidids mean that we are not able to pinpoint the centre of origin of the heteronchocleidids. However the monophyly of the heteronchocleidids and the presence of the heteronchocleidids on these two groups of fish hosts suggest that the two host groups must have evolved in the same place and time.

There are also other *Channa* species, for example *Channa micropeltes* (Cuvier & Valenciennes) and *C. gachua* (Hamilton-Buchanan), where no heteronchocleidids have been recorded thus far (Lim & Furtado, 1985; Lim, personal observation). The absence of heteronchocleidids on the two *Channa* spp. could be explained by loss through failure to speciate and hence extinction of the heteronchocleidids on these channids. The presence of *Sundanonchus* spp. on *C. micropeltes* (see Lim & Furtado, 1985; Krtisky & Lim, 1995) indicates that *C. micropeltes* is either very different from the other channids (Lim, 1997) or that *C. micropeltes* was able to capture a new monogenean species, probably from a *Pristolepis* sp. (Lim & Furtado, 1985). The latter is more probable since *C. micropeltes* has been shown to be closely related to the other channids (Fig. 3). The above indicate that the phenomena of species extinction and host transfer cannot be ignored as factors in host-monogenean distribution pattern.

The present host-heteronchocleidid distribution pattern is probably the results of a combination of inheritance with or without host transfer within and between the anabantoids and channids early in their evolutionary history. Subsequent speciation, failure to speciate and extinction (Johnson et al., 2003) of the heteronchocleidids within their hosts could result in the survival of only one group of heteronchocleidids on each of the anabantoid and channid species. The early divergence of the *Trianchoratus* from the *Heteronchocleidus-Eutrianchoratus* and grouping of species within *Trianchoratus* clade according to their channid and anabantoid hosts, suggest that speciation of the *Trianchoratus* had occurred within their respective fish host groups. As noted above more data are needed, especially on the interrelationships of the heteronchocleidids to other monogenean groups for a clearer picture of the evolutionary history of the heteronchocleidids.

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